Natural and commercial *Salix* clones differ in their ecophysiological response to Zn stress

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Abstract

This study was carried out to determine the effect of different zinc concentrations on the ecophysiological response of *Salix* clones: four commercial clones ("1962", "1968", "Drago", and "Levante") selected for short rotation coppice, and one natural clone, "Sacco", obtained from a contaminated area. Gas exchanges, chlorophyll *a* fluorescence (JIP-test), relative chlorophyll content, and biometric parameters were measured in plants grown for fifteen days in soil containing Zn concentrations of 0, 300, 750, and 1,500 mg($ZnCl_2$) kg^{-1}. Ecophysiological response to metal stress differed in dependence on the Zn concentration and clone. At the low Zn concentration (300 mg kg^{-1}), the absence of any significant reductions in parameters investigated indicated an efficient plant homeostasis to maintain the metal content within phytotoxic limits. Stomatal limitation, observed at 750 and 1,500 mg kg^{-1}, which was found in all clones after three days of the treatment, might be caused by indirect effects of metal on guard cells. Among commercial clones, "Drago" was more sensitive to Zn stress, showing inhibition of growth, while "1962" clone showed a downregulation of PSII photochemistry following the slowdown in the Calvin-Benson cycle. On the contrary, the natural *Salix* clone ("Sacco") performed better compared to the other clones due to activation of a photosynthetic compensatory mechanism.

Additional key words: chlorophyll *a* fluorescence; gas exchange; stress response; willow; zinc.

Introduction

In natural environments, heavy metals are present at low concentrations without causing significant toxic effects to living organisms. However, their increased accumulation in soil and water may have serious consequences for plants, animals, and human health. In Europe, it has been estimated that heavy metals and mineral oils contribute to about 60% of soil contamination and 53% of the groundwater contamination (Panagos *et al.* 2013). The main anthropogenic sources of heavy metals occur due to human activities, such as mining, extensive use of fertilizers, and sewage production. In the last years, the potential of some tree species in removing metal ions from soil has received more and more attention. In particular, *Salicaceae* are considered good candidates for a phytoremediation approach (Marmiroli *et al.* 2011) due to their easy propagation, high tolerance against metal

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Abbreviations: C_i – substomatal CO_2 concentration; DAT – days after treatment; DM – dry mass; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximal fluorescence yield of the dark-adapted state; FM – fresh mass; g_s – stomatal conductance; LA – leaf area; PI_{abs} – performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; PI_{total} – performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; P_N – net assimilation rate; RC/CS_0 – number proportional to the active RCs to the cross-section of the measured sample; RH – relative air humidity; RuBP – ribulose-1,5-bisphosphate; SLA – specific leaf area; SRC – short rotation coppice; ΔV_{I-P} – amplitude of the I–P phase of the OJIP fluorescence transient; φ_{P0} – maximal quantum yield of PSII photochemistry; Ψ_0 – probability that a photon trapped by the PSII RC enters the electron transport chain.

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pollutants, and high annual biomass production, which may be used in the energy supply chain by means of short rotation coppice cultures (SRC) (Zacchini *et al.* 2009). Until today, many studies have evaluated effects on the ability of absorption and translocation to plant organs and effects at the molecular level under different growth conditions, suggesting a large genetic variability between species (Pulford and Watson 2003, Konlechner *et al.* 2013). In particular, some authors have observed that some willows are tolerant to zinc and tend to accumulate the metal in the aboveground biomass (Landberg and Greger 1996, Lunáčkova *et al.* 2003, Rosselli *et al.* 2003, Mleczek *et al.* 2009) including leaves (Migeon *et al.* 2009, Dos Santos Utmazian *et al.* 2007).

In response to metal stress, plants exhibit morphological and metabolic changes, such as growth inhibition and changes in physiological and biochemical response (Solanki *et al.* 2011, de Silva *et al.* 2012, Shah *et al.* 2010). Among micronutrients, Zn plays an important physiological role: it is essential for both plants and animals but, when it reaches high concentrations in tissues, it can lead to the production of reactive oxygen species (ROS) and the consequent oxidative stress (Lin *et al.* 2012). The Zn excess can reduce the absorption of micronutrients, such as Fe, Mg, and P, change antioxidant enzyme activities, and can cause the alteration of the ultrastructure of chloroplasts, as well as the reduction of the CO_2 assimilation and alteration of photochemical processes (Tsonev *et al.* 2012). The negative action of Zn on photosynthesis is well documented in plants (Di Baccio *et al.* 2003, Sagardoy *et al.* 2009), but its effects on the light phase of photosynthesis are controversial at high concentrations.

In some studies, the maximum quantum yield of PSII (φ_{P0}) was not affected in Zn-treated plants (Dhir *et al.* 2008, Sagardoy *et al.* 2009, Salvatori *et al.* 2014), while other studies have shown the inhibition of thylakoid electron transport by high Zn concentrations (Kim and Jung 1993). Moreover, the effects on PSII and PSI are often dose-dependent (Babu *et al.* 2010) and increase with the duration of exposure (Chugh and Sawhney 1999). Khan *et al.* (2007) analyzed the impact of heavy metals on the electron transport system in isolated chloroplasts and found that PSII is more susceptible than PSI. However, only recently it has been observed that also the PSI activity can be inhibited by high concentrations of metals (Wodala *et al.* 2012, Belatik *et al.* 2013).

The accumulation of heavy metals in plant species may induce effects at physiological level (Prasad 2004). A set of different ecophysiological parameters, as gas exchanges and chlorophyll fluorescence (JIP-test), may be used to assess metal stress *in vivo* (Ernst and Peterson 1994, Ayeni *et al.* 2010).

The aim of the present study was to investigate the ability of different *Salix* clones to withstand low (300 mg kg^{-1}), medium (750 mg kg^{-1}), and high (1,500 mg kg^{-1}) zinc concentrations in soil under controlled environmental conditions. The ecophysiological response of natural and commercial willow clones was analyzed to evaluate the effects of the Zn treatment on the photosynthetic process by the analysis of CO_2 assimilation and chlorophyll (Chl) *a* fluorescence (JIP-test). The following hypothesis were tested: (*1*) the different ability to withstand high Zn contents in soil solution exists among clones, and (*2*) *S. alba* cuttings, coming from a contaminated area, are less sensitive to zinc than other clones selected to produce high biomass at natural sites.

Materials and methods

Plant material, growth conditions and Zn treatments: The experiment was carried out in a "walk-in" chamber (for further details about experimental set-up *see* Salvatori *et al.* 2013), under the following climatic conditions: day-night temperature of 24–19°C (T_a), relative humidity of 65–70% (RH), photoperiod of 14 h and maximum PAR of 500 µmol m^{-2} s^{-1} at plant height. The experimental plant material consisted of five willow clones:

Salix alba – clone SI068-015 "1968";
Salix alba – clone SI062-019 "1962";
Salix mutsudana ×? (open pollination) – clone S76-004 "Drago";
Salix mutsudana ×? (open pollination) – clone S76-005 "Levante";
Salix alba – clone "Sacco".

The first four clones, used for SRC, were supplied by the Istituto Sperimentale per la Pioppicoltura (Casale Monferrato, Italy), while the "Sacco" clone was derived from the natural growing population at a contaminated area, the "Sacco River Valley" (Latium Region, Italy), which was subjected to industrial pollution for several years. In particular, in this area, both water and soil pollution were caused by chemical, mechanical, electronic, and food industrial factories, mainly located in the municipalities of Colleferro, Anagni, Frosinone, and Ceccano, resulting in local accumulation of toxic waste dumps. Furthermore, many areas in proximity of the river are highly anthropized, being occupied by intensive farming and agricultural systems, warehouses, houses and roads, further contributing to enhancement of pollution levels through chemical fertilizers and pesticides, municipal discharges, animal wastes, and sewage irrigation. Accordingly, since 2005, the existence of an environmental and socio-economic emergency in the Sacco River valley has been recognized and both water and soils have been monitored (Report Activities Lazio Region 2011). Moreover, the analysis carried out on both blood of exposed residents and on bovine milk, has highlighted a worrying contamination by organochlorine compounds and heavy metals (Report DEP Lazio 2013, Battisti *et al.* 2013, Sala *et al.* 2012). In particular, the zinc concentrations in plants (SWS 2013) and in topsoils (Report ISPRA 2009) of agricultural riparian areas were detected close to the limit values (113.46 and 230 mg kg^{-1}, respectively).

Woody cuttings (25 cm long) of equivalent size were grown in plastic pots (1.5 L) containing a 2:1 garden soil and sand (pH 7.1) in a completely randomized block design. After two months of acclimation in growth chamber, three plants per clones were exposed to metal concentrations: 0 (control), 300, 750, and 1,500 mg(Zn) kg^{-1}(soil). The lower concentration (300 mg kg^{-1}) has been chosen on the base of previous studies carried out on poplar clones exposed to zinc stress in laboratory experiments (Lingua *et al.* 2008, Todeschini *et al.* 2011, Durand *et al.* 2011). The medium concentration (750 mg kg^{-1}) exceeds optimal concentration in leaves and may induce visible toxicity symptoms (Marshener, 1997), while the higher concentration (1,500 mg kg^{-1}) is in accordance with the limit value of Legislative Decree 152/2006 "Norms Concerning the Environment", commonly called "Single Environmental Text" for Italian soils in industrial areas. Zinc was supplied as $ZnCl_2$ and the experiment lasted for 15 d.

Gas exchange: Net photosynthetic rate (P_N), stomatal conductance (g_s), and substomatal CO_2 concentration (C_i) were measured after 3, 10, and 15 d (DAT) from the Zn treatment by a portable infrared gas analyzer (*Ciras 2*, *PP System*, UK). Measurements were made between 10:00 and 12:00 h, on the first two fully developed leaves from the top of the plant, and were repeated on three plants for each treatment ($n = 6$).

Chl *a* fluorescence: The Chl *a* fluorescence parameters were measured by using a *Handy PEA*, plant efficiency analyzer (*Hansatech*, King's Lynn, Norfolk, UK), on the same leaves measured for gas exchanges after 15 DAT. Leaves were dark-adapted for 30 min and the fast fluorescence transient was measured by applying a saturating red light pulse of 3,000 μmol(photon) m^{-2} s^{-1} during 1 s. When plotted on a logarithmic time-scale, this fast fluorescence signal exhibits a series of steps between the initial O (F_0, when all the reaction centers of the PSII are open) and maximum P level (F_m, when all the PSII reaction centers are fully reduced). The JIP-test is a tool that translates this polyphasic fluorescence transient into a constellation of biophysical parameters that quantify the single steps of the photochemical pathway through both PSII and PSI (Strasser *et al.* 2010). The software *Biolyzer* (*Bioenergetics Lab.*, Geneva, CH) was used to calculate the JIP-test parameters (Strasser 2004, Strasser *et al.* 2010). In particular, the following parameters were considered:

$RC/CS_0 = \varphi_{P0}\ (V_J/M_0)\ F_0$. It is a measure for Q_A^- reducing RCs per excited leaf cross-section.

$\Psi_0 = ET_0/TR_0 = 1 - V_J$. It expresses the efficiency with which a trapped photon can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors.

PI_{abs} – performance index on absorption basis. It is the performance index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors. It combines three parameters directly linked to photosynthetic activity: the density of reaction centers (expressed on an absorption basis, RC/ABS), the quantum yield of primary photochemistry ($\varphi_{P0} = TR_0/ABS$), and the ability to feed electrons into the electron chain between PSII and PSI ($\Psi_0 = ET_0/TR_0$).

$\Delta V_{I\text{-}P} = 1 - V_I = (F_m - F_{30\,ms})/(F_m - F_0)$. It indicates the amplitude of the I–P phase, *i.e.* the electron transport efficiency around PSI to reduce the final acceptors of the electron transport chain (ETC), *i.e.* ferredoxin and NADP (Oukarroum *et al.* 2009);

PI_{total} – total performance index. It is the performance index for energy conservation of photons absorbed by PSII, through the ETC, to the reduction flux (RE) of PSI end acceptors (formulae from Strasser *et al.* 2010).

Relative Chl content and biometric parameters: At the end of the experiment, for each Zn treatment, the relative Chl content was measured by using a portable chlorophyll meter (*SPAD*, *Minolta*, Osaka, Japan) on the first two fully developed leaves from the top of each plant and the results were expressed as "SPAD units". Fifteen leaves for each clones and treatment were sampled for growth analysis. The samples were weighed for fresh mass (FM) and, after oven drying at 60°C until constant mass, for dry mass (DM). Leaf area (LA) was measured on fresh leaves using a scanner and an image analysis software (*Image Lab, Version 2.2.4.0, MCM Design*), and specific leaf area (SLA) (corresponding to LA/DM, cm^2 g^{-1}) was calculated. The growth was estimated as difference between the stem length (SL) measured after 15 DAT and before the addition of metal.

Statistical analysis: Given the non-normal distribution and the lack of equality of the variances of the data set, data were analyzed using the non-parametric *Kruskal-Wallis* analysis of variance (*ANOVA*) test and *post-hoc* comparisons of mean ranks of all pairs of groups (Siegel and Castellan 1988). Differences were considered as significant at $p \le 0.05$ and highly significant at $p \le 0.001$. All statistical procedures were performed by using *Statistica v 7.0* software (*StatSoft, Inc.*, Tulsa, OK, USA). Data in figures are expressed as the percentage variation respect to the control plants.

Results

Gas exchange: The Zn effect on g_s was evident after 3 DAT in all clones (Fig. 1*B*) at the medium concentration (750 mg kg^{-1}), in particular, in Levante that showed a significant inhibition of P_N (–43% with respect to control plants, Fig. 1*A*) and a reduction of C_i (–24%, Fig. 1*C*), indicating that stomatal limitation to photosynthesis was occurring. At the high Zn concentration (1,500 mg kg^{-1}) the reduction of P_N was more pronounced in 1962 and 1968 (80% and 85% of control, respectively) than that in Levante, Drago, and Sacco (respectively, 51, 37, and 36% lower than control, Fig. 1*A*) due to inhibition of g_s. Only in Levante, a reduction of C_i was observed (–32% compared with control plants, Fig. 1*C*).

After ten days of exposure, 1968, Sacco, Drago, and Levante clones showed a recovery of gas exchange while 1962 highlighted a reduction of P_N and g_s (respectively, of –42% and 60% at 750 mg kg^{-1} and 60% and 67% at 1,500 mg kg^{-1} compared with control plants, Fig. 1*D*,*E*). Moreover, at the end of treatment (15 DAT), P_N increased in Sacco at 300 mg kg^{-1} and 1,500 mg kg^{-1} (56% and 42% with respect to control, Fig. 1*G*), while g_s and C_i values remained significantly inhibited in 1962 clone (–48% and –22% compared with the control plants, respectively, Fig. 1*H*,*I*).

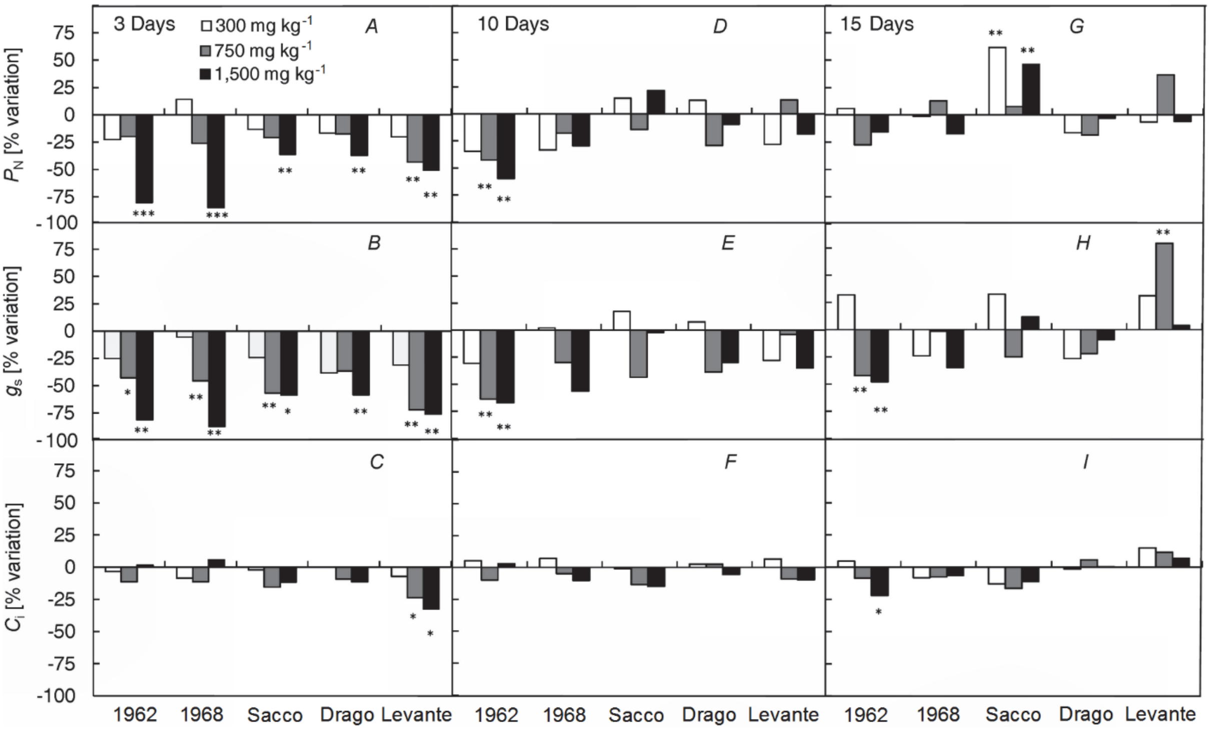

Fig. 1. Effects of Zn concentrations on the net photosynthetic rate (*A,D,G*), stomatal conductance (*B,E,H*), and substomatal CO_2 concentration (*C,F,I*) in willow clones. Plants were cultivated with Zn concentrations equal to 300, 750, and 1,500 mg kg^{-1} for 3, 10, and 15 days. P_N – net photosynthetic rate, g_s – stomatal conductance, C_i – substomatal CO_2 concentration. Values express the percentage variation with respect to the control plants (n = 6). Asterisks indicate significant difference from control plants: * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$.

Fluorescence analysis: In all clones after 15 DAT, the fluorescence parameters did not show any significant difference from controls in the plants treated with the low Zn concentration (300 mg kg^{-1}) (Fig. 2). Significant differences, compared with the controls, were observed in 1962 and Sacco clones at the higher concentrations. In particular, at 750 mg kg^{-1}, $\Delta V_{I\text{-}P}$ and PI_{total} decreased in 1962 (Fig. 2*D*,*F*), while the parameter indicating electron transport probability (Ψ_0) increased in Sacco (Fig. 2*C*). As a consequence, PI_{abs} and PI_{total} were significantly enhanced by Zn in this clone (Fig. 2*E*,*F*).

The increase of basal fluorescence, F_0, and the reduction of the number of active RCs per leaf cross-section (RC/CS_0) in 1962 (Fig. 2*A*,*B*) indicated a marked effect of the high Zn concentration (1,500 mg kg^{-1}); it affected also Ψ_0, giving a significant reduction of $\Delta V_{I\text{-}P}$, PI_{abs}, and PI_{total}. In the Sacco clone, the photochemical response was opposite: F_0 decreased, while Ψ_0 increased, followed by an increase of PI_{abs} and PI_{total}.

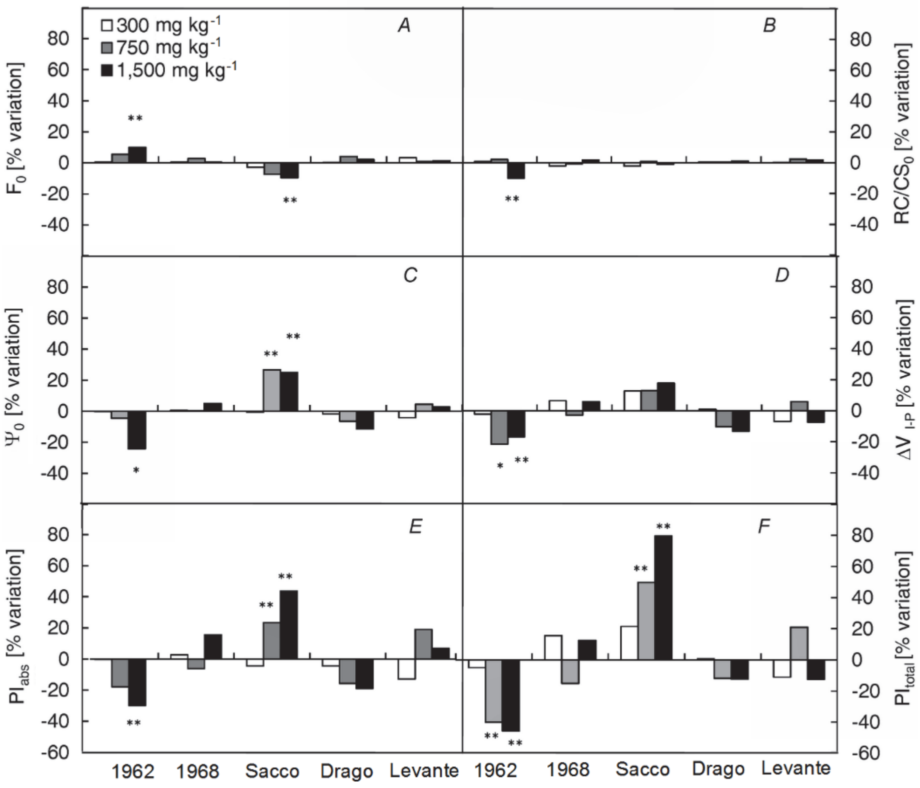

Fig. 2. Values of chlorophyll *a* fluorescence parameters derived by the JIP-test: minimal fluorescence, F_0, (*A*); a number proportional to the active RCs to the cross-section, RC/CS_0, (*B*); electron transport probability, Ψ_0, (*C*); the amplitude of the I–P phase, $\Delta V_{I\text{-}P}$, (*D*); performance index on absorption basis, PI_{abs}, (*E*), and total photosynthetic performance index, PI_{total}, (*F*) in willow clones exposed to Zn: 300, 750, and 1,500 mg kg^{-1} for 15 days. Values express the percentage variation with respect to the control plants ($n = 6$). Asterisks indicate significant difference from the control plants: * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$.

Relative Chl content and biometric parameters: The effects of different Zn concentrations on the relative Chl content, leaf area (LA), and SLA in different clones are shown in Table 1. No significant difference was observed at the low and medium Zn concentration (300 and 750 mg kg^{-1}), while the Zn treatment negatively affected relative pigment contents in leaves at 1,500 mg kg^{-1} in 1962 after 15 DAT (–24% with respect to the control plants). Also LA was markedly inhibited at the high Zn concentration (1,500 mg kg^{-1}) in 1962, whereas it was not affected by other Zn treatments and in other clones. SLA showed equal values with respect to the control plants at different Zn concentrations in each clone (Table 1). The growth was stimulated in 1968 and Sacco at 300 mg kg^{-1} and in Levante at 750 mg kg^{-1}, while it decreased in Drago at the end of the treatments, (–48% with respect to control at the high Zn concentration, Fig. 3).

Discussion

In plants, the photosynthetic apparatus is very sensitive to the toxicity of Zn and it affects the assimilation process both directly, by inhibiting the Calvin cycle enzyme activities, and indirectly, by inducing CO_2 deficiency at the carboxylation sites due to stomatal closure (Shah *et al.* 2010).

In this study, the low Zn concentration (300 mg kg^{-1}) added into soil was below the phytotoxic limits (Marshner 1997) and did not altered the gas exchange and the photochemical activity, as the result of an efficient metal homeostasis. Plants posses, in fact, homeostatic mechanisms to maintain the proper concentrations of essential metal ions in different cellular compartments and to minimize the damage from exposure to excessive metal ions (Clemens 2001). Our results are in agreement with Durand *et al.* (2011), who did not observe any inhibition of stomatal conductance and assimilation rate in a poplar hybrid clone (*Populus tremula* × *P. alba*) under similar Zn exposure. In short term, the inhibitory effects of excessive Zn on photosynthetic processes were observed in all clones at medium and high concentrations (750 and 1,500 mg kg^{-1}). The results from hybrid *Populus deltoids* × *Populus nigra* (Di Baccio *et al.* 2003), *S. viminalis*, and *P. tremula* (Hermle *et al.* 2006) were comparable to *Salix* clones

Table 1. Effect of different Zn concentrations on the relative total chlorophyll content (*n* = 6), expressed in SPAD units, leaf area (LA, *n* = 15), and specific leaf area (SLA, *n* = 15) were analyzed at the end of treatments (15 days). Values are expressed as mean ± SE. Asterisks indicate significant difference from control plants: * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$. C – control.

Clone	$ZnCl_2$ [mg kg^{-1}]	Total chlorophyll	LA [cm^2]	SLA [cm^2 g^{-1}]
1962	C	42.700 ± 1.470	19.047 ± 0.803	185.607 ± 9.475
	300	37.950 ± 1.704	18.462 ± 1.449	193.732 ± 9.424
	750	38.183 ± 0.893	17.487 ± 0.414	184.841 ± 5.435
	1,500	32.350 ± 1.573***	12.810 ± 0.723**	173.391 ± 13.331
1968	C	34.750 ± 1.632	13.410 ± 2.541	174.840 ± 9.513
	300	38.771 ± 2.965	14.070 ± 3.211	155.954 ± 9.675
	750	31.883 ± 1.413	12.195 ± 2.073	176.971 ± 14.969
	1,500	32.967 ± 0.957	8.873 ± 0.701	186.133 ± 6.239
Sacco	C	39.367 ± 2.205	6.479 ± 0.729	164.210 ± 12.452
	300	37.429 ± 2.014	6.386 ± 0.540	147.044 ± 8.405
	750	40.817 ± 3.018	6.468 ± 0.677	164.332 ± 12.752
	1,500	41.333 ± 1.630	7.454 ± 0.239	195.504 ± 20.413
Drago	C	32.367 ± 1.673	17.501 ± 0.822	177.199 ± 14.658
	300	30.400 ± 1.686	18.503 ± 1.515	211.228 ± 15.859
	750	33.167 ± 2.663	14.493 ± 0.728	176.408 ± 12.915
	1,500	35.300 ± 2.204	14.355 ± 0.464	198.451 ± 8.975
Levante	C	43.300 ± 1.049	13.627 ± 0.646	187.775 ± 10.138
	300	36.967 ± 2.013	12.691 ± 1.939	171.484 ± 12.823
	750	41.150 ± 3.063	14.724 ± 0.706	179.504 ± 11.474
	1,500	43.817 ± 2.020	10.082 ± 0.563	149.064 ± 16.290

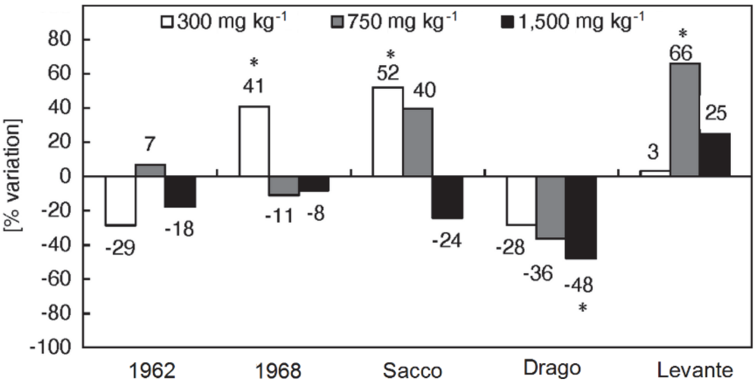

Fig. 3. Growth in willow clones exposed to various concentrations of Zn, estimated as difference between the stem length (SL, cm) after 15 days of treatment and before the addition of metal. Values express the percentage variation with respect to the control plants (*n* = 3). Asterisks indicate significant difference from the control plants: * – $p \leq 0.05$; ** – $p \leq 0.01$; *** – $p \leq 0.001$.

investigated in this experiment, with the decline in leaf gas exchange parameters after treatment with excessive Zn concentrations. The stomatal closure observed together with Zn supply may be due to the inhibition of water channels that affect stomatal movements (Yang *et al.* 2004) or due to an indirect effects of Zn on guard cell, linked to the reduction of K^+ uptake, which alters the ratio between K^+ and other ions (Tsala Ndzono *et al.* 1994, Vaillant *et al.* 2005). In 1962 clone under the high Zn concentration, stomatal reduction and reduced CO_2 assimilation persisted even after 15 d, suggesting a significant metal accumulation in leaves and a lower detoxification capacity for Zn.

The decline of fluorescence parameters in the connection with the excess of heavy metal was evidenced by several authors (Adamaski *et al.* 2011, Wang *et al.* 2012, Xue *et al.* 2013, Kalaji *et al.* 2014). Concerning Zn, it is known that all components of the photosynthetic apparatus (*e.g.* the pool of plastoquinone, the oxygen evolving complex, PSII and PSI) can be altered or damaged by this metal (Prasad 2004). In particular, under Zn excess, the structure of the thylakoid membranes may be affected by the metal, with a consequent reduction of the electron transport rates. Moreover, the inactivation of Chl *a* due to the Mg^{2+} replacement with Zn^{2+}, as well as the substitution of Mn^{2+} with Zn^{2+} at the site of water photolysis, has been also reported (Santos *et al.* 2014). In our case, in 1962 cultivar, the Zn excess (1,500 mg kg^{-1}) reduced the energy transfer from PSII-Chl *a* antenna to the PSII reaction center (increase of F_0) and the number of active RCs per leaf cross-section (decrease of RC/CS_0). However, the reduction of the probability (Ψ_0) of the linear photosynthetic electron transport between PSII and PSI, as well as of the photosynthetic performance index on absorption basis (PI_{abs}), indicated a down regulation of the PSII photochemistry, concurrently to the marked Zn-induced reduction of gas exchange. This downregulation mechanism helps maintain the electron flux in equilibrium with the decreased NADPH and ATP demand of the Calvin cycle limiting the probability of the formation of reactive oxygen species. Moreover, the I-P part of the fluorescence transient revealed that Zn stress also affected PSI activity and content. The inhibition of ΔV_{I-P}, a parameter sensitive to abiotic stress factors, such as drought (Pollastrini *et al.* 2014, Oukarroum *et al.* 2009), ozone (Mereu *et al.* 2011, Salvatori *et al.* 2013, Desotgiu *et al.* 2013), and nitrogen availability (Nikiforou and Manetas 2011), and the decline of PI_{total} represented a lower efficiency of electron transport around PSI in order to reduce the final acceptors of the electron transport chain. The amplitude of I-P phase seems to be depressed as a consequence of a lower request of reductants, which limits the efficiency in carbon reduction and the turnover of ribulose-1,5-bisphosphate (RuBP) (Strasser *et al.* 2010), suppressing the Calvin-Benson cycle due to the closure of stomata. Our results can be interpreted as a response of the clones to the different accumulation of Zn. All clones, except 1962, may have took only a subtoxic amount of Zn from the soil and modulated their metabolism in order to cope with this stress. This is a typical physiological response of the plants that are less sensitive to the metal stress.

Furthermore, the high energy dissipation in the antenna pigments (decrease of F_0), the increase of $\square_{\square}$ and the consequent increase of photosynthetic performance (PI_{abs} and PI_{total}) in Sacco, could represent a photosynthetic compensatory mechanism through an increased efficiency of carboxylation and RuBP regeneration capacity under Zn stress (at 750 mg kg^{-1} and 1,500 mg kg^{-1}), as observed by Pollastrini *et al.* (2014) and Ugolini *et al.* (2014) in other species. The increase of P_N at the end of the treatment allowed us to surmise that this clone might have limited the phytotoxicity of the metal in aerial organs by activating detoxification and compartmentalization mechanisms. Therefore, this strategy might help the Sacco clone perform better with respect to other clones and be able to withstand better Zn stress due to increase of photosynthetic activity in presence of the metal within a short term.

Chl content declines in leaves of plants under heavy metal stress and thus plants diminish their efficiency to absorb light (Papazoglou *et al.* 2007). In our case, the alteration of photochemical reactions, observed in leaves of 1962 at the 1,500 mg kg^{-1} concentration after 15 DAT, might result from the decrease in the Chl content (reduction of relative pigments content was 24% with respect to control); it suggests a possible damage to the Chl synthesis in thylakoids (Küpper *et al.* 1998, Di Baccio *et al.* 2009, Shi *et al.* 2009). In other clones, the measured Chl content remained unaffected and no significant differences were observed between the Zn-treated and control plants. This finding indicated that the metal was probably sequestered in a very effective manner within the plant, thus providing a protection of the photosynthetic machinery, supported by detoxification mechanisms, as observed also by Mittler *et al.* (2004).

The effect of the Zn treatment was also emphasized in the plant growth, which was different for each clone and concentration. The lower Zn concentration promoted the growth in 1968 and Sacco at 300 mg kg^{-1} and in Levante at 750 mg kg^{-1}, probably by acting as a fertilizer, while in Drago, despite the recovery of gas exchange, Zn stress inhibited the growth. This may be due to the reduction of stem or/and root dry mass, as observed by Di Baccio *et al.* (2009) in *Populus* × *euramericana*, clone I-214. Root growth is a more sensitive endpoint marker of metal toxicity than Chl contents; its inhibition is often due to the accumulation of Zn in roots in order to limit the metal translocation to leaves, which are more sensitive to metals (Morgan *et al.* 2002). However, in agreement with Wang *et al.* (2009), at root level, the Zn excess in soil might also cause an imbalance in nutritional uptake and limited photoassimilate translocation to leaves. Perhaps, these events were induced in the 1962 clone, that showed the reduction of leaf area index (LA) in addition to the limitation of gas exchange and photosynthetic performance at the high Zn concentration. The reduction of the Chl content and biometric parameters, such as photosynthesizing leaf area and stem length, are a general responses of higher plants to heavy metal toxicity (Nagajyoti *et al.* 2010) and represent indirect effect of metals (Agarwal *et al.* 2012).

In conclusion, our present results allowed to discriminate a gradient of the functional response among natural and commercial *Salix* clones, from the least sensitive to the most sensitive clone:

- at low Zn concentration (300 mg kg^{-1}):
Sacco≥1968=1962=Drago=Levante

- at medium Zn concentration (750 mg kg^{-1}):
Levante>Sacco=Drago=1968=1962

- at high Zn concentration (1,500 mg kg^{-1})
Levante>Sacco=1968=1962>Drago

Moreover, in agreement with our initial hypothesis, the five considered willow clones exhibited a different ecophysiological response to increasing Zn concentrations in soil; Sacco clone showed the highest ability to withstand metal stress compared to the commercial clones selected to produce high biomass. In fact, this clone, obtained from a natural population growing in a contaminated area and adapted to Mediterranean climatic conditions, appeared to be better performing among the *Salix* clones under the Zn stress condition. Therefore, we propose to investigate it more and under field conditions to assess its potential in phytoremediation technologies.

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